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Amphotericin B Derivative Blocks Human Immunodeficiency Virus Type 1 Entry after CD4 Binding: Effect on Virus-Cell Fusion but Not on Cell-Cell Fusion

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The antiviral effect of MS8209, an amphotericin B derivative, was studied in CD4$^+$ cells transfected with a *lacZ* gene inducible upon human immunodeficiency virus type 1 (HIV-1) infection. MS8209 was shown to block virus entry after receptor binding and probably before virus-cell membrane fusion, but it had no effect on syncytium formation, although both processes are mediated by HIV-1 envelope proteins and CD4.

MS8209 is a derivative of the antifungal agent amphotericin B, with improved water solubility and reduced toxicity to cells and animals (27). Micromolar concentrations were shown to prevent human immunodeficiency virus type 1 (HIV-1) replication in CD4$^+$ lymphocytes and cell lines in the absence of detectable cytotoxicity (7). The mechanism of action of MS8209 was studied in the CD4-positive HeLa P4 cell line that had been stably transfected with a *lacZ* gene inducible by the HIV-1 Tat protein (9). Early expression of Tat allows the detection of HIV-1-infected P4 cells, after a single cycle of virus replication, by an in situ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) assay (15, 26).

P4 cells were grown in the presence of MS8209, at concentrations ranging from 0.1 to 100 μM, without a significant effect on their proliferation (Fig. 1). The number of infected (blue-stained) cells was determined after overnight contact with the HIV-1_{NDK} strain (33). Infection was blocked by 5 μM MS8209 or higher concentrations, the 50 and 90% inhibitory concentrations being 1 and 2.5 μM, respectively (Fig. 1). The efficacy of the drug in this assay indicated an antiviral action on either HIV-1 entry, reverse transcription, integration, or early gene expression, since later steps of the HIV-1 replicative cycle were not addressed. To determine which step was blocked, MS8209 was added to P4 cells either before, during, and/or after contact with HIV-1, and parallel experiments were performed with zidovudine (AZT), a reverse transcriptase inhibitor (22, 29). Both drugs blocked infection when maintained throughout the experiment but had markedly different time-of-addition requirements for optimal activity (Fig. 2). To block infection, MS8209 had to be present during the phase of virus-cell contact and was unnecessary afterwards, whereas AZT acted in the hours after virus addition, as expected. MS8209 therefore blocked an earlier step of HIV-1 replication than AZT, probably virus entry. Also, previous in vitro experiments indicate it did not interfere with reverse transcription (7). The target of MS8209 does not seem to be a cellular component, since there was a limited antiviral effect when the drug was present before virus-cell contact (Fig. 2). Unexpectedly, we observed the same effect with AZT. Cells could absorb a subinhibitory amount of drug during the pretreatment, but a side effect of AZT on HIV-1 entry cannot be ruled out, since it was shown to reduce the formation of syncytia between uninfected CD4$^+$ cells and HIV-infected cells (6).

HIV-1 enters cells by pH-independent fusion with the plasma membrane, a complex process initiated by the binding of the viral surface glycoprotein, gp120, to its receptor, CD4 (reviewed in references 23, 32, and 34). The antiviral action of MS8209 is probably not exerted at this initial step, since it did not prevent binding of recombinant gp120 to CD4$^+$ cells (7) or to soluble CD4 (data not shown), unlike other drugs blocking HIV-1 entry, such as dextran sulfate (2, 21). Furthermore, we could show that MS8209 blocked the entry of already-bound HIV-1 to CD4$^+$ cells. For that, P4 cells were left in contact with virus for 1 h at 4°C to allow binding but not membrane fusion (18), then washed to remove unbound virus, and transferred to 37°C to allow entry to proceed. A complete inhibition of infection was seen when MS8209 was added immediately before the temperature shift or 5 min later, and about 80 and 50% inhibition was seen when it was added 15 and 30 min later, respectively (Fig. 3). This experiment showed that MS8209 blocked a step of HIV-1 entry immediately following CD4 binding, possibly membrane fusion. Photoinactivation experiments have shown that membrane fusion takes place in the minutes following CD4 binding (12), consistent with the kinetics of action of MS8209.

Fusion of the viral lipidic envelope with the cell membrane is usually addressed indirectly, by syncytium formation assays between CD4$^+$ cells and cells expressing HIV-1 envelope proteins (Env gp120 and Env gp41). We have performed cocultures of P4 cells with HeLa-env cells (30), which are stably transfected with an *env* expression vector and express surface gp120/gp41 and Tat. Syncytia formed with P4 cells can therefore be quantitated by X-Gal staining. There was no inhibition of syncytium formation by MS8209, even when a small number of HeLa-env cells was used (Table 1) or in the presence of 100 μM MS8209 (Fig. 4), a concentration 10-fold higher than the 50% inhibitory concentration in infectivity assays. Although fusion with CD4$^+$ cells is very efficient, gp120 is barely detectable at the surfaces of HeLa-env cells (30) and the lack of effect of the drug on cell-to-cell fusion is unlikely to be due to a higher amount of Env proteins. The lack of effect of MS8209 in these experiments was not unexpected since the parental drug, amphotericin B, favors cell-to-cell fusion mediated by other viral envelopes (34) and was recently shown to allow fusion of cells expressing HIV-2 envelope proteins with CD4$^+$ murine cells (19).

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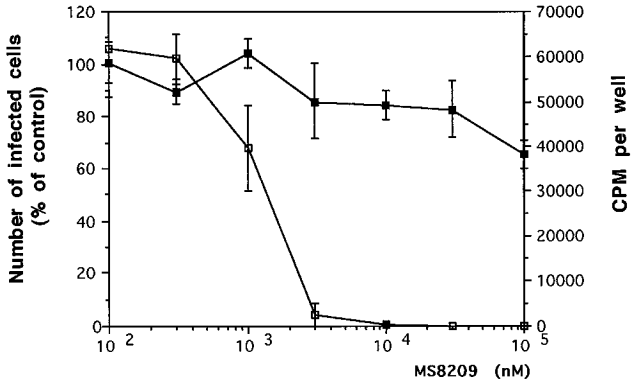

FIG. 1. Effect of MS8209 on HIV-1 infection and cell proliferation. CD4$^+$ HeLa P4 cells were grown to ~50% confluency in 96-well plates and infected with HIV-1_{NDK} in the presence of the indicated concentration of MS8209. Virus and drug were left in contact with cells for 16 h before X-Gal assay. Open squares represent the average number of blue-stained cells per well (mean of six wells) plotted as a percent of the untreated control cells (~100 blue cells). Vertical bars represent the standard error of mean. Proliferation of P4 cells (solid squares) was measured by [^{3}H]thymidine uptake after 24 h of contact with drug.

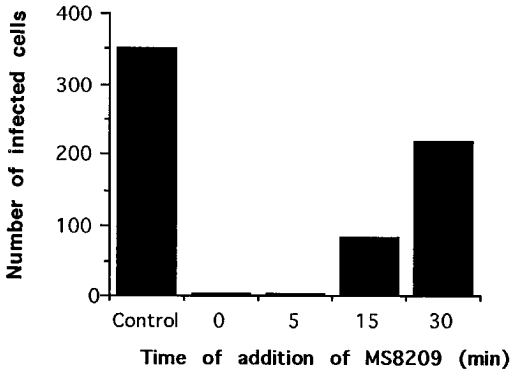

FIG. 3. Antiviral effect of MS8209 on HIV-1 bound to CF4$^+$ cells. P4 cells (subconfluent 12-well plates) were incubated with HIV-1_{NDK} at 4°C for 1 h to allow virus binding but not entry. Unbound virus was removed by two washes in phosphate-buffered saline at 4°C, and plates were transferred to 37°C. MS8209 (final concentration, 10 μM) was added at the indicated times after the temperature shift and left in contact with cells. The number of blue-stained cells was scored by X-Gal assay 16 h after infection. The means of two experiments are shown.

On the basis of the assumption that virus-cell and cell-cell fusion had the same mechanism, drugs blocking HIV-1 entry but not syncytium formation, such as bicyclams (10) or hypericin (11, 17, 20), have been proposed to act on a postfusion step of virus entry termed uncoating. However, this step cannot be assayed directly, and its viral and cellular requirements are not defined. In the case of HIV-1 strains adapted to T-cell lines, virus-cell and cell-cell fusion are indeed highly related processes. Syncytia are only formed in CD4$^+$ cells permissive to HIV-1 entry (8), and mutations in gp120 or gp41 that reduce their ability to induce cell fusion also reduce virus infectivity (4, 13, 16). But the viral and cellular requirements of HIV-1 entry and syncytium formation may not be totally identical. Neutralizing antibodies against the third variable domain (V3) of gp120 are less efficient on syncytium formation (24). Heparin blocks HIV-1 entry but not syncytium formation (2, 3) and was recently shown to inhibit the binding of antibodies to V3 but not the gp120-CD4 interaction (14). Also, cellular factors not required for virus entry, such as the LFA-1 adhesion molecule, could influence the ability of CD4$^+$ cell lines to form syncytia (25).

We therefore considered that an action of MS8209 on virus-cell fusion could not be ruled out from its lack of action in syncytium formation assays. We sought to address the postbinding steps of HIV-1 entry in an indirect assay, on the basis of the recently observed property of HIV-1 particles to induce fusion from without of CD4$^+$ cells in the absence of viral replication (9). Fusion from without was detected and quantitated by X-Gal assay, after the addition of HIV-1 to a coculture of P4 cells (CD4$^+$ *lacZ*$^+$) and Jurkat-tat cells (CD4$^+$ Tat$^+$) in the presence of 5 μM AZT to block HIV-1 replication and prevent de novo expression of gp120/gp41 (Fig. 4; Table 2). Although the readout of this assay is the fusion of a Tat$^+$ and a *lacZ*$^+$ cell, an initial step of virus-cell fusion is required. The number of blue-stained foci detected by this assay was dramatically reduced in the presence of 10 μM MS8209 (Fig.

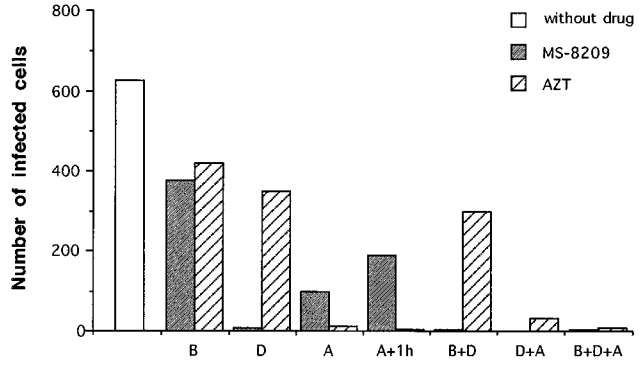

FIG. 2. Time requirements for inhibition of HIV-1 infection by MS8209 and AZT. P4 cells were treated either with 10 μM MS8209 (filled bars) or with 1 μM AZT (hatched bars) for 1 h before infection (B), during a 1-h incubation with HIV-1_{NDK} (D), immediately after this incubation (A), or 1 h later (A+1h). Incubations with virus and/or drugs were performed at 37°C and ended with two washes in phosphate-buffered saline. In treatments A and A+1h, drugs were left in contact with cells. X-Gal assays were performed 16 h after infection. The means of two independent experiments are shown.

TABLE 1. Effect of MS8209 on fusion between P4 and HeLa-env cells

Fusion of CD4$^+$ *lacZ* cells[a] and indicated no. of Env$^+$ Tat$^+$ cells[b]	MS8209 (μM) (final concn)	No. of blue syncytia/well[c]
10^5		11,635
10^5	1	13,250
10^5	10	12,800
10^5	100	12,550
10^4		8,910
10^4	1	8,255
10^4	10	9,170
10^4	100	7,860
10^3		655
10^3	1	917
10^3	10	755
10^3	100	524

[a] 10^5 P4 cells per well (12-well plate).
[b] HeLa-env cells per well.
[c] X-Gal assays were performed after overnight coculture. Results are from one representative experiment.

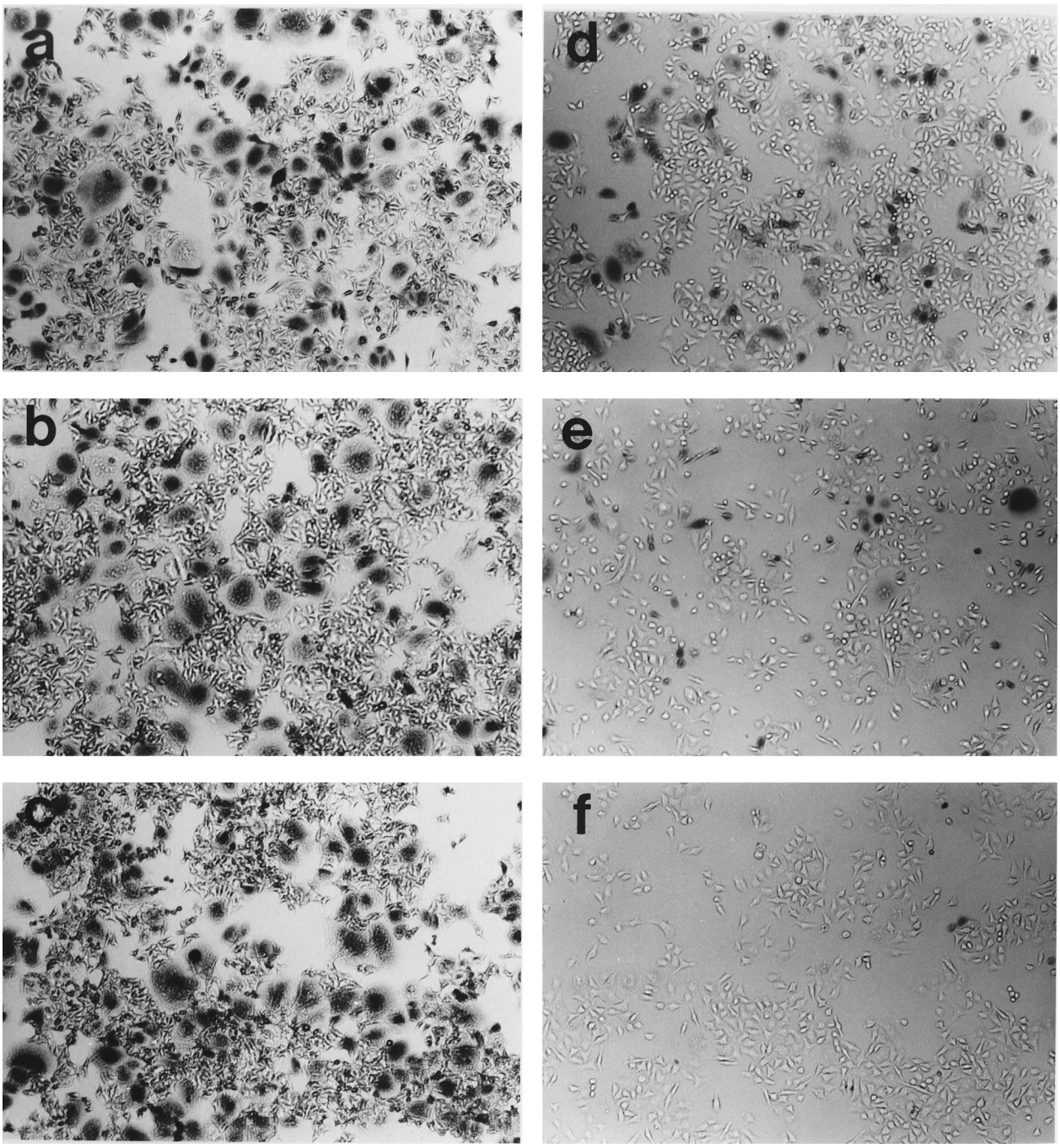

FIG. 4. Effect of MS8209 on cell-cell and virus-cell fusion mediated by the HIV-1 envelope. Cocultures of P4 cells and HeLa-env cells, which express HIV-1 gp120/gp41, were either untreated (a) or treated with 10 μM (b) or 100 μM (c) MS8209. P4 and HeLa-env cells were trypsinized, mixed in a 1:1 ratio, and allowed to adhere to 12-well plates in the presence of drug when indicated. X-Gal assays were performed after overnight coculture. (d to f) Fusion from without of P4 cells and Jurkat-tat cells by HIV-1_{NDK} in the absence of drug (d), in the presence of 5 μM AZT (e), or in the presence of 5 μM AZT and 10 μM MS8209 (f). About 250,000 Jurkat-tat cells were added to a subconfluent monolayer of P4 cells (100,000 cells per well). Drugs were added prior to virus (~5,000 IU). X-Gal assays were performed 16 h after virus addition.

4; Table 2), suggesting that it blocked an early step of HIV-1 entry that was not required for syncytium formation.

Further evidence that the drug was active before membrane fusion could be the loss of infectivity of viral stocks after incubation with 10 μM MS8209 at 37°C but not at 4°C (Fig. 5). The infectious titer was not restored by ultracentrifugation, suggesting either direct HIV-1 inactivation by the drug or its stable interaction with viral particles. Treating viral stocks with 10 μM MS8209 for 1 h was not sufficient for a complete loss of infectivity (Fig. 5), although the same concentration of drug was fully active when added during virus-cell contact (Fig. 2).

It is likely that MS8209 associates with membranes, since the parental drug amphotericin B is known to modify membrane permeability by interaction with cholesterol (5). The lipid

TABLE 2. Effect of MS8209 on the fusion from without of $CD4^+$ *lacZ* P4 cells and Jurkat-tat cells by HIV-1

$CD4^+$ Tat^+ cells[a]	HIV-1_{NDK}	AZT (5 μM)	MS8209 (10 μM)	No. of blue cells/well[b]
−	+	−	−	4,250
−	+	+	−	45
−	+	−	+	20
+	−	−	−	0
+	+	−	−	8,050
+	+	+	−	3,600
+	+	+	+	60

[a] 10^5 Jurkat-tat cells per well.
[b] 10^5 P4 cells per well (12 well-plate). X-Gal assays were performed 16 h after infection. Results are from one representative experiment.

bilayer enveloping HIV was found to contain higher quantities of cholesterol than the plasma membrane of uninfected or virus-producing cells (1). The effect of MS8209 on virus-cell fusion but not on cell-cell fusion could be due to uptake of higher amounts of drug by viral membranes. However, Env^+ cells could be grown permanently in high concentrations of drug without detectable loss of fusion with $CD4^+$ cells. Also, MS8209 had absolutely no antiviral effect on HIV-2 or on HIV-1 pseudotyped with other retroviral envelope proteins (unpublished data). We are therefore led to conclude that the antiviral effect of MS8209 is exerted, at least in part, on HIV-1 envelope proteins. The incomplete inactivation of HIV-1 after cell-free incubation with MS8209 could suggest an interaction with a domain more accessible after CD4-gp120 interaction, such as V3 in gp120 (32). We previously mentioned that other anti-V3 agents blocked virus entry but had little or no effect on syncytium formation.

MS8209 may therefore be useful to define the early steps of HIV-1 entry and their viral and cellular requirements. Amphotericin B and its derivatives, as well as nystatin A, are structurally related to MS8209 and also exert an antiviral effect on HIV-1 (28, 31). Although a detailed study of their mechanisms of action has not yet been reported, our preliminary experiments indicate a mode of action similar to that of MS8209. A comparative study may be useful to define the chemical basis of the antiviral effect of this group of agents and to derive new, more efficient therapies.

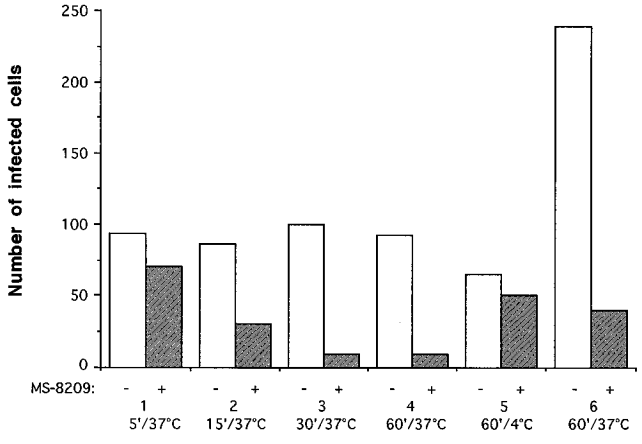

FIG. 5. Direct effect of MS8209 on HIV-1 infectivity. A cell-free HIV-1_{NDK} stock was incubated at 37°C or 4°C for the indicated times in the absence (open bars) or in the presence (filled bars) of 10 μM MS8209. The virus titer was measured by the infection of P4 cells as described in the legend to Fig. 1, with the final concentration of MS8209 being 0.5 μM. In experiment 6, virus was pelleted by ultracentrifugation after incubation with or without drug and then resuspended in fresh medium before infection of P4 cells. An increased virus titer was observed after this treatment.

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